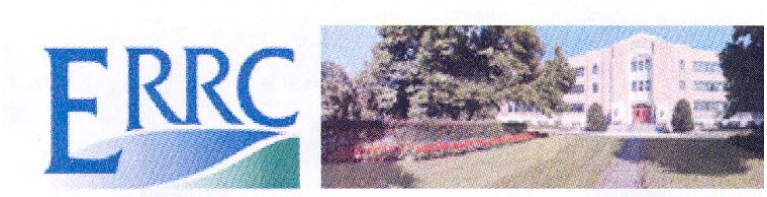

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Efficacy of Different Washing Agents in Reducing the Populations of *Escherichia coli* O157:H7 on Artificially Inoculated Golden Delicious Apples

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Conventional and experimental washing formulations were applied at 20 and 60°C to determine their efficacy in decontaminating apples inoculated with *Escherichia coli* O157:H7. Treated and untreated apple samples were analyzed for residual *E. coli* O157:H7 using whole and dissected apple samples. *E. coli* O157:H7 was able to infiltrate the core of the apple, mainly through the calyx region. *E. coli* O157:H7 populations were mainly attached to the calyx and stem regions compared to the outer skin of the apple. Apples were washed in a covered glass tank containing one of the following washing solutions (4 liters): tap water, 5% hydrogen peroxide, 1200 ppm Sanova, 850 ppm Sanova, 500 ppm Sanova, 850 ppm Sanova basic, 850 ppm Sanova acidic, 400 and 200 ppm chlorine (pH=8.8), and 400 and 200 ppm chlorine (pH=6.5). None of the washing treatments used under the conditions of this experiment was able to completely inactivate *E. coli* O157:H7 populations on the inoculated apples, probably as a consequence of the inability of this washing system to inactivate or remove the bacterial cells in inaccessible calyx, stem and core regions of the apple. Washing at 60°C resulted in higher log reduction in the pathogen populations when compared to washing at 20°C. These results are important because they demonstrate the need for new fruit washing technologies that can overcome the inability to decontaminate the calyx and stem regions of apples.

1. Introduction

Since 1991 there have been several outbreaks of foodborne disease associated with *Escherichia coli* O157:H7 in unpasteurized apple cider. These outbreaks were particularly significant, as they occurred in a highly acidic food product, previously thought to be safe because of its low pH (3.3-3.5). However, studies have shown that *E. coli* O157:H7 can survive in unpasteurized apple cider produced in the traditional manner. The fact that this is a ready-to-eat product, receiving no further processing before consumption, should be a matter of concern. As a consequence of these outbreaks the Food and Drug Administration (FDA), has mandated that juice products be treated with a process designed to yield a 5 $\log_{10}$ reduction in the most resistant organisms of public concern. Products not treated in this way must carry a warning label informing consumers of the potential risk of foodborne disease associated with the product. The presence of pathogens such as *E. coli* O157:H7 on the surface of fruit also has implications for safety of supplies to the fresh and fresh-cut fruit markets.

As a result of the FDA ruling, there has been considerable research effort expended in the development of intervention methods by which to achieve the target 5 $\log_{10}$ reduction during the processing of juice products. A recent survey of small scale apple cider producers, (those producing less than 5000 gallons of cider annually), indicated that 80% of producers would be interested in using alternative technologies to help assure the safety of their products. Potential intervention methods include pasteurization, both UV and thermal, irradiation, use of anti-microbial treatments such as ozone, high pressure inactivation and/or modified storage procedures.

Although, heat pasteurization is the most reliable method used to reduce pathogen numbers by 5 $\log_{10}$ in juice products, only 22% of small-scale apple cider producers pasteurize their product. The capital expenditure required for the purchase of the necessary equipment, in addition to adverse changes to sensory characteristics of the product, are among the reasons cited for the low pasteurization rate. Identification of alternative, less expensive intervention methods would be of great benefit to juice processors, and fresh and fresh-cut fruit markets.

In this study, the efficacy of conventional and experimental sanitizing agents as well as the washing temperature in reducing *E. coli* O157:H7 populations on artificially inoculated apples were investigated. Also, we investigated the limitation of these washing treatments in reducing inaccessible and/or internalized *E. coli* O157:H7 populations.

2. Materials and Methods

Microorganism maintenance. *Escherichia coli* strain SEA 13B88 (from an outbreak associated with apple cider in the northwest United States in1996) was obtained from Dr. Pina Fratamico (USDA-ERRC, Wyndmoor, PA), and stored in 20% glycerol at – 80°C. The organism was transferred monthly in tryptic soy broth (TSB; Difco, Detroit, MI. USA) and maintained on tryptic soy agar (TSA; Difco) at 4°C.

Inoculum development, growth conditions and apple inoculation. A loop full of culture from TSA plate was transferred into 10 ml of TSB and allowed to grow for approximately 8 h at 37°C. This culture was then used to inoculate 1 liter of the same medium at a 0.01 % (v/v) level. The culture was allowed to grow for 18 h at 37°C, spun down at 9500 x *g* for 5 min, washed once with 200 ml sterile distilled water, and suspended in 2 liters of tap water to a final cell concentration of approx 9 log CFU/ml. Unwaxed Golden Delicious apples, free of decay and punctures, were immersed in the 2 liters inoculum (5 apples at once) for 5 min with occasional shaking. Apples were drained, placed on their sides in a plastic bin to permit drainage from stem and calyx areas, and allowed to dry at 4°C overnight (ca. 20 h).

Washing protocol and washing agents. Composites of 4 inoculated apples were washed in an 8 liter covered glass jar containing 4 liters of washing solutions, at 20 or 60°C, with shaking for 2 min, using water bath with shaker.. Apples were briefly drained prior to sample preparation (see below) for assessment of residual bacterial viability on whole apples, and calyx, stem and core portions of apples.

The washing agents used in this study were as follows: **A)** water; **B)** 200 or 400 ppm (wt/v) chlorine, prepared from sodium hypochlorite; **C)** 200 or 400 ppm (wt/v) chlorine, prepared from sodium hypochlorite and adjusted to pH 6.5 with hydrochloric acid; **D)** 5% (v/v) hydrogen peroxide (H_2O_2; EKA Chemicals, Marietta, GA); **E)** 1200, 850 or 500 ppm acidified sodium chlorite (Sanova), was prepared by mixing equal amounts of basic and acidic solutions prior to washing step according to the manufacturer's guidelines (Alcide Corporation, Redmond, WA. USA)

Sample preparation: a) Preparation of whole blend samples: duplicate composites of 4 pieces of fruit each were blended with an equal volume (w/v) of 0.1% (w/v) peptone (Difco) water (PW). (b) Preparation of stem, core and calyx homogenates: two composites of 4 apples each were used for this procedure. Stem, calyx and core pieces of each fruit were aseptically removed, and the remainder of the fruit was discarded. Duplicate sets of 4 stem, core or calyx portions were individually blended in 4 parts (w/v) PW.

Blended duplicate sets were filtered through a stomacher filter bag and 30 ml fruit filtrate (2 X 15 ml duplicate samples) from each blended set was collected. Fruit filtrates (1 or 0.1 ml aliquots) were diluted as necessary in PW and plated (see below), with the remainder of the filtrate retained at 4°C.

Assessment of bacterial viability. The bacterial suspensions were serially diluted in PW and surface plated on duplicate CTSMAC plates. CTSMAC was prepared using sorbitol MacConkey agar (Difco) supplemented with 0.05 and 2.5 mg/l of cefexime and tellurite (Dynal, Lake Success, NY. USA), respectively. The plates were then incubated at 37°C for 18-24 h, and the survivors were enumerated. Cell densities were reported as log CFU per gram of sample.

3. Results

Efficacy of washing treatments in reducing total *E.* coli O157:H7 population. The effect of washing agents and washing temperature on log reduction of *E.* coli o157:H7 on whole apples is shown in Table 1. Apples were inoculated with an average of 5.4 log CFU/gm (Table 1). There was a maximum of 3.4 log reduction in *E. coli* O157:H7 population on whole apples using 200 ppm chlorine solution (pH=6.5) at 60°C. 200 ppm chlorine solution resulted in higher log reduction when compared to 400 ppm chlorine solution. Adjusting the pH of the 200 ppm chlorine solution to 6.5 resulted in higher log reduction in the cell population of *E.* coli O157:H7 as compared to non-adjusted pH=8.8 solution (Table 1).

1200 and 850 ppm Sanova solutions resulted in a comparable log reduction as compared to 500 ppm solution at 20 or 60°C (Table 1). The Sanova basic and acidic solutions (850 ppm) resulted in up to 1.3 less log reduction in *E. coli* O157:H7 population when compared to the 850 ppm mixed solution (Table 1).

Washing trials at 60°C consistently resulted in higher cell reduction as compared to washing temperature at 20°C (Table 1), except when 400 ppm chlorine solution (pH=8.8) was the washing solution.

The use of washing agents resulted in up to 1.6 more log reduction compared to when water was the only washing agent (Table 1).

Efficacy of washing treatments in reducing *E. coli* O157:H7 populations in calyx and stem regions. The effect of washing conditions on cell population of *E. coli* O157:H7 in calyx and stem

(inaccessible external regions) of apples, are shown in Tables 2 and 3, respectively. Calyx and stem regions of the apple were inoculated with approximately 6 log CFU/gm of *E. coli* O157:H7 (Tables 2-3). Standard deviation for samples treated with 850 ppm Sanova solution was not calculated (Table 2) since more than one determination showed no growth on CTSMAC medium. In contrast to the results obtained with whole apple blends (Table 1),
Washing at 60°C resulted at higher log reduction in *E. coli* O157:H7 population in the calyx and stem regions compared to washing at 20°C (Tables 2-3). Hydrogen peroxide, 1200 and 850-ppm Sanova solutions resulted in 2-4 more log reduction in *E coli* O157:H7 counts when compared to the other washing solutions used (Tables 2-3).

Efficacy of washing treatments in reducing internalized *E. coli* O157:H7 population. The effect of washing treatment on log reduction in internalized *E. coli* O157:H7 cell population in the core region of apples is shown in Table 4. The inoculated control showed a 4 log CFU/gm of *E. coli* O157:H7 in the core region of apples (Table 4). Standard deviation for tap water and hydrogen peroxide treatments at 20°C were not calculated (Table 4) since more than one determination showed no growth on CTSMAC medium. The increase in washing temperature did not correspond to a substantial increase in the log reduction in *E. coli* O157:H7 population in the core region of apples (Table 4). 200 ppm chlorine solution (pH 8.8 and 6.5) showed the highest log reduction when compared to the other washing agents used (Table 4).

4. Summary and Conclusions

♦ Total *E.* coli O157:H7 was 7.6 log CFU/apple using the whole blend. The combined total cell concentration in the calyx and stem areas was 7.1 log CFU/apple. This indicated that *E.* coli O157:H7 cells on artificially inoculated apples were mainly attached to stem and calyx areas compared to the outer skin of the apple.

♦ Increase in the washing temperature corresponded to an increase in the log reduction in *E. coli* O157:H7 populations in whole, calyx and stem blends. Washing temperature did not reduce the internalized bacterial load in the core region. This could be due to the fact that the core region is well insulated as compared to the skin, calyx and stem areas of the apple. This indicated that the internalized pathogenic cells are inaccessible to the antimicrobial wash.

♦ When compared to tap water as the washing solution, all washing agents tested here resulted in higher log reduction in the *E. coli* O157:H7 populations on apples, except for Sanova basic and acidic solutions (Table 1). This indicates that a sanitizing agent should always be used during all washing processes of apples, to reduce the microbial load and to prevent any possible cross contamination of the fruit.

♦ No washing treatment resulted in complete reduction in the internalized *E. coli* O157:H7 in the core of the apple (Table 4). This indicates that further treatment of the juice products should be mandated for the production of safe food product.

♦ Some washing treatment resulted in up to 6 log reduction in *E. coli* O157:H7 populations in the calyx, stem and core regions. It was too difficult to consistently inactivate pathogenic cells in those regions using the current washing method. New technology that improves the contact between the inaccessible and/or internalized pathogenic organisms and the antimicrobial wash, accomplishes pasteurization, or physically removes these pathogenic organisms from calyx, stem and core regions of the apple, is required.

Table 1. Effect of washing treatment on log reduction[A] in total *Escherichia coli* O157:H7 cell concentration on whole apples.

	Washing Temperature	
Washing Reagent	20°C (log_{10} CFU/gm)	60°C (log_{10} CFU/gm)
Tap Water	1.54 ± 0.06	1.84 ± 0.14
Hydrogen Peroxide (5% solution)	2.36 ± 0.17	2.71 ± 0.15
Sanova[B] (1200 ppm)	2.74 ± 0.41	2.75 ± 0.19
Sanova[B] (850 ppm)	2.33 ± 0.27	2.75 ± 0.84
Sanova[B] (500 ppm)	1.87 ± 0.22	2.18 ± 0.21
Sanova base[C] (850 ppm)	1.25 ± 0.12	2.53 ± 0.12
Sanova acid[D] (850 ppm)	1.01 ± 0.14	2.01 ± 0.31
Chlorine (400 ppm, pH=8.8)	2.31 ± 0.28	2.25 ± 0.10
Chlorine (400 ppm, pH[E]=6.5)	1.05 ± 0.28	2.08 ± 0.47
Chlorine (200 ppm, pH=8.8)	2.24 ± 0.31	2.57 ± 0.44
Chlorine (200 ppm, pH[E]= 6.5)	2.72 ± 0.30	3.38 ± 0.25
Inoculated Control[F]	5.35 ± 0.25	

[A] Data reported as log reduction in cell concentration ± standard deviation, following a washing treatment.

[B] Sanova (acidified sodium chlorite) solution was prepared by mixing basic and acidic solutions (1:1) according to the manufacturer's protocol.

[C] Sanova base was prepared by mixing basic solution with deionized water (1:1).

[D] Sanova acid was prepared by mixing acidic solution with deionized water (1:1).

[E] The pH of the chlorine solution was adjusted to 6.5 using concentrated Hydrochloric acid.

[F] Mean population of *E. coli* O157:H7 detected on inoculated, untreated whole apple samples (32 composites of 4 apples each).

Table 2. Effect of washing treatment on log reduction[A] in *Escherichia coli* O157:H7 cell concentration in the calyx region of apples.

	Washing Temperature	
Washing Reagent	20°C (log_{10} CFU/gm)	60°C (log_{10} CFU/gm)
Tap Water	1.16± 0.18	1.72 ± 0.10
Hydrogen Peroxide (5% solution)	2.09 ± 0.02	4.29 ± 1.30
Sanova[B] (1200 ppm)	3.82 ± 0.01	NG[C]
Sanova[B] (850 ppm)	3.37[D]	NG[C]
Sanova[B] (500 ppm)	2.43 ± 1.60	2.67 ± 1.84
Sanova base[E] (850 ppm)	1.00 ± 0.16	1.38 ± 0.25
Sanova acid[F] (850 ppm)	1.21 ± 0.19	2.36 ± 0.17
Chlorine (400 ppm, pH=8.8)	1.71 ± 0.69	2.29 ± 0.88
Chlorine (400 ppm, pH[G]=6.5)	1.46 ± 0.11	1.89 ± 1.11
Chlorine (200 ppm, pH=8.8)	0.70 ± 0.42	1.94 ± 1.44
Chlorine (200 ppm, pH[G]= 6.5)	1.03 ± 0.45	0.96 ± 2.66
Inoculated Control[H]	5.84 ± 0.86	

[A] Data reported as reduction in cell concentration ± standard deviation, following a washing treatment.

[B] Sanova (acidified sodium chlorite) solution was prepared by mixing basic and acidic solutions (1:1) according to the manufacturer's protocol.

[C] NG = No growth was detected.

[D] No standard deviation was reported since three out of four replicates resulted in no growth.

[E] Sanova base was prepared by mixing basic solution with deionized water (1:1).

[F] Sanova acid was prepared by mixing acidic solution with deionized water (1:1).

[G] The pH of the chlorine solution was adjusted to 6.5 using concentrated Hydrochloric acid.

[H] Mean population of *E. coli* O157:H7 detected on inoculated, untreated calyx samples (29 composites of 4 calyces).

Table 3. Effect of washing treatment on log reduction[A] in *Escherichia coli* O157:H7 cell concentration in the stem region of apples.

	Washing Temperature	
Washing Reagent	20°C (log_{10} CFU/gm)	60°C (log_{10} CFU/gm)
Tap Water	1.21 ± 0.06	3.91 ± 0.12
Hydrogen Peroxide (5% solution)	2.40 ± 0.22	3.51 ± 0.86
Sanova[B] (1200 ppm)	3.87 ± 0.37	4.03 ± 0.48
Sanova[B] (850 ppm)	3.04 ± 0.63	NG[C]
Sanova[B] (500 ppm)	2.87 ± 0.30	2.39 ± 0.20
Sanova base[D] (850 ppm)	1.78 ± 0.12	2.44 ± 0.62
Sanova acid[E] (850 ppm)	0.96 ± 0.15	1.95 ± 0.36
Chlorine (400 ppm, pH=8.8)	1.71 ± 0.69	2.29 ± 0.88
Chlorine (400 ppm, pH[F]=6.5)	0.91 ± 0.15	2.42 ± 0.30
Chlorine (200 ppm, pH=8.8)	1.54 ± 0.41	2.84 ± 0.12
Chlorine (200 ppm, pH[F]=6.5)	3.06 ± 0.05	1.96 ± 0.24
Inoculated Control[G]	6.08 ± 0.25	

[A] Data reported as reduction in cell concentration ± standard deviation, following a washing treatment.

[B] Sanova (acidified sodium chlorite) solution was prepared by mixing basic and acidic solutions (1:1) according to the manufacturer's protocol.

[C] NG = No growth was detected.

[D] Sanova base was prepared by mixing basic solution with deionized water (1:1).

[E] Sanova acid was prepared by mixing acidic solution with deionized water (1:1).

[F] The pH of the chlorine solution was adjusted to 6.5 using concentrated Hydrochloric acid.

[G] Mean population of *E. coli* O157:H7 detected on inoculated, untreated core samples (26 composite of 4 stems).

Table 4. Effect of washing treatment on log reduction[A] in *Escherichia coli* O157:H7 cell concentration in the core region of apples.

	Washing Temperature	
Washing Reagent	20°C (log_{10} CFU/gm)	60°C (log_{10} CFU/gm)
Tap Water	0.79[B]	1.48 ± 0.09
Hydrogen Peroxide (5% solution)	0.22[B]	0.83 ± 0.05
Sanova[C] (1200 ppm)	2.24 ± 0.12	2.34 ± 0.14
Sanova[C] (850 ppm)	NG[D]	1.04 ± 0.01
Sanova[C] (500 ppm)	2.24 ± 1.17	1.01 ± 0.36
Sanova base[E] (850 ppm)	1.45 ± 1.18	1.86 ± 0.97
Sanova acid[F] (850 ppm)	1.02 ± 0.39	1.63 ± 0.74
Chlorine (400 ppm, pH=8.8)	2.98 ± 0.47	2.29 ± 0.01
Chlorine (400 ppm, pH[G]=6.5)	2.67 ± 0.03	1.42 ± 0.45
Chlorine (200 ppm, pH=8.8)	2.80 ± 1.85	3.30 ± 1.29
Chlorine (200 ppm, pH[G]= 6.5)	1.64 ± 0.06	3.37 ± 1.75
Inoculated Control[H]	3.99 ± 0.67	

[A] Data reported as reduction in cell concentration ± standard deviation, following a washing treatment.

[B] No standard deviation was reported since three out of four replicates resulted in no growth.

[C] Sanova (acidified sodium chlorite) solution was prepared by mixing basic and acidic solutions (1:1) according to the manufacturer's protocol.

[D] NG = No growth was detected.

[E] Sanova base was prepared by mixing basic solution with deionized water (1:1).

[F] Sanova acid was prepared by mixing acidic solution with deionized water (1:1).

[G] The pH of the chlorine solution was adjusted to 6.5 using concentrated Hydrochloric acid

[H] Mean population of *E. coli* O157:H7 detected on inoculated, untreated core samples (26 composite of 4 cores).